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Patentanmeldung Nr. Patent application No. Demande de brevet n°

99114853.7

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 99114853.7
Demande n°:

Anmeldetag:
Date of filing: 29/07/99
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
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Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Neuroprotective properties of GDF-15 in vitro and in vivo

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:	Tag:	Aktenzeichen:
State:	Date:	File no.
Pays:	Date:	Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:

/

Am Anmeldetag benannte Vertragsstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du dépôt:

Bemerkungen:
Remarks:
Remarques:



Applicant: Biopharm Gesellschaft zur biotechnologischen Entwicklung von
Pharmaka mbH
"Neuroprotective properties of GDF-15 in vitro and in vivo"
Our Ref: B 1951EU - py / js

EPO - Munich
52

29. Juli 1999

Description

The present invention relates to a transforming growth factor-beta (TGF- β)-like protein which is derived from neurons and glial cells, and which has a neurotrophic effect on dopaminergic (DAergic) neurons, to nucleic acids coding for the protein, to a vector containing the nucleic acids, to host organisms containing the nucleic acids or the vector, to antibodies directed against the protein, to methods for the production of the nucleic acids, the vector or the protein, to a pharmaceutical composition for the treatment of neurodegenerative disorders in mammals and to a diagnostic kit for the detection of said disorders.

- 10 Members of the TGF- β superfamily are known for their important multifunctional implications in development and maintenance, such as the organization of the body plan, regulation of cell proliferation, differentiation, and cell survival. The still expanding TGF- β superfamily includes the TGF- β isoforms 1 to 5, activins, inhibins, bone morphogenetic proteins (BMPs), growth/differentiation factors
- 15 (GDFs), mullerian-inhibiting substance, *Drosophila* decapentaplegic gene complex, *Xenopus* Vg-1 gene, and a growing subfamily of glial cell line-derived growth factors (GDNFs) and related proteins. All members of the TGF- β superfamily share several homologous structures. They are synthesized as large precursor molecules containing a biologically inactive pro-domain which can be
- 20 secreted as a complex with the mature carboxyterminal portion. Furthermore, the mature bioactive proteins are generated by proteolysis using a characteristic cleavage site. Most notably, the mature carboxy-terminal segments contain a highly conserved cysteine knot.
- 25 As TGF- β -like proteins are also implicated in the regulation of neuronal stem cell proliferation and maintenance of neurons there is a great demand for novel members of this protein family.

Accordingly, the technical problem underlying the present invention is to provide novel compounds relating to TGF- β -like proteins having neurotrophic activities which are suitable for the treatment and diagnosis of neurodegenerative disorders.

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The solution to the above technical problem is achieved by providing the embodiments as characterized in the claims.

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In particular, the present invention relates to a nucleic acid containing a nucleotide sequence encoding the primary amino acid sequence of a TGF- β -like protein or a functionally active derivative or part thereof which is derived from neurons and glial cells and which has a neurotrophic effect on DAergic neurons.

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The terms "nucleic acid" and "nucleotide sequence" refer to endogenously expressed, semi-synthetic, synthetic or chemically modified nucleic acid molecules, preferably consisting substantially of deoxyribonucleotides and/or ribonucleotides and/or modified nucleotides. Further, the term "nucleotide sequence" may comprise exons, wherein the nucleotide sequence encodes the primary amino acid sequence and may be degenerated based on the genetic code. The term "primary amino acid sequence" refers to the sequence of amino acids irrespective of tertiary and quaternary protein structure.

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The term "TGF- β -like protein" refers to proteins displaying the characteristics of the TGF- β superfamily, especially a conserved cysteine rich motif, and comprises both the large precursor molecules containing a pro-domain as well as the mature bioactive proteins which are generated by proteolysis using a characteristic cleavage site.

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The terms "functionally active derivative" and "functionally active part" refer to a proteinaceous compound exhibiting at least a neurotrophic effect on DAergic neurons. The functionally active form of the above-defined TGF- β -like protein may be a monomeric, dimeric and/or oligomeric form, as well as a heterooligomeric form, e.g. a heterodimer, comprising at least two different monomers of

TGF- β -like proteins having neurotrophic activity.

The expression "derived from neurons and glial cells" means that the gene coding for the protein is transcribed and/or translated in neurons and glial cells such as Purkinje cells and astrocytes such that the mRNA and/or the protein is detectable by methods known in the art such as *in situ* hybridization, RT-PCR, Northern or Western blotting.

The expression "neurotrophic effect on DAergic neurons" refers to a proteinaceous activity that may confer, by itself or in combination with other factors, survival and differentiation upon DAergic neurons within the nanomolar range or below.

In a preferred embodiment of the above defined nucleic acid the neurons and glial cells are of mammalian origin, e.g. human, mouse or rat.

In a further preferred embodiment, the TGF- β -like protein protects against neurodegenerative events. Such neurodegenerative events may be e.g. mediated by oxidative damage, free radicals, mediators or executors of neuronal death programs such as caspases, pro- and anti-apoptotic members of the bcl-2 family. A toxic radical damage may be mediated by iron, e.g. Fe-ions, NO and other radical donors. Therefore, the nucleic acid as defined above encodes a TGF- β -like protein which is able to protect DAergic neurons against intoxication by iron, which is suggested to cause Parkinson's disease (PD).

In a further preferred embodiment the nucleic acid according to the present invention comprises at least the nucleotide sequence shown in Fig. 5A or the nucleotide sequence shown in Fig. 6A or nucleotides 40 to 333 of the nucleotide sequence shown in Fig. 6A or mutants of such nucleic acids leading to the expression of functionally active polypeptides. Examples of such mutations include deletions, insertions and substitutions of one or more nucleotides such as mutations which lead to conservative amino acid substitutions, e.g. such mutations in the range of nucleotides 40 to 333 of the nucleotide sequence

shown in Fig. 6A, i.e. the region of the nucleotide sequence encoding the 7 Cys-knot region which is highly conserved in TGF- β -like proteins.

5 A further subject of the present invention relates to a vector containing at least the nucleic acid as defined above. The term "vector" refers to a DNA and/or RNA replicon that can be used for the amplification and/or expression of the above defined nucleotide sequence. The vector may contain any useful control units such as promoters, enhancers, or other stretches of sequence within the 5' regions of the sequence serving for the control of its expression. The vector
10 may additionally contain sequences within the 5' and/or 3' region of the nucleotide sequence, that encode amino acid sequences such as a His-tag which are useful for the detection and/or isolation of the protein encoded by the nucleotide sequence. Furthermore, the vector may contain sequence elements within the 5' and/or 3' region of the nucleotide sequence encoding amino acid sequences
15 which serve for the targeting of the protein encoded by the nucleotide sequence to nerve tissues and/or for the penetration of the blood/brain barrier. Examples of suitable vectors are baculovirus vectors.

20 Another embodiment of the present invention relates to a host organism containing the nucleic acid or the vector, as defined above. The term "host organism" comprises a virus, a bacterium such as *Escherichia coli*, a fungus, a plant, a mammal or an insect or parts such as cells, e.g. Sf9 cells, thereof.

25 A further embodiment of the present invention relates to the protein itself, which is encoded by the nucleic acid as defined above. Examples of the primary amino acid sequence of the protein according to the present invention are given in Figs. 5B and 6B, respectively. Further examples of the primary amino acid sequence of the protein according to the present invention comprise amino acid residues 14 to 111 of the sequence shown in Fig. 6B as well as homologs thereof having
30 conservative amino acid substitutions.

A further subject of the present invention relates to an antibody, which may be monoclonal or polyclonal, or a functional fragment thereof directed against the

protein or a functional derivative or part thereof as defined above. Further subjects of the present invention relate to an antagonist directed to the above-defined protein and to an agonist as a substitute for the above-defined protein.

- 5 A modulation of the functional activity of the above-defined protein may also be achieved by altering the expression of the nucleotide sequence of the above-defined nucleic acid as compared to the expression level in a normal cell. For example, an antisense nucleic acid masking the mRNA or a ribozyme cleaving the mRNA may be used to inhibit the expression. Alternatively, the efficiency of
- 10 the promoter which regulates the expression of the nucleotide sequence of the above-defined nucleic acid may be influenced.

- A further embodiment of the present invention relates to a method for the production of the nucleic acid, the vector, or the protein as defined above, comprising the steps of:
- 15

- (a) cultivating the above-defined host organism in a suitable medium under suitable conditions; and
- (b) isolating the desired product from the medium and/or the host organisms.

- 20 A preferred embodiment of the method for the production of the protein according to the present invention uses bacteria such as *E. coli* as the host organism. The expression of the above-defined protein may then lead to a functionally inactive form, e.g. of amorphous aggregates within the bacterium known in the art as "inclusion bodies". Therefore, the method of the present invention may
- 25 further comprise steps serving for the refolding and/or modification of the isolated protein into a functionally active form which may be a monomeric, dimeric or oligomeric form.

- A further embodiment of the present invention relates to a pharmaceutical composition comprising the nucleic acid or the vector or the protein or the antibody or the antagonist or the agonist as defined above, optionally in combination with a pharmaceutically acceptable carrier and/or diluent. The pharmaceutical composition may be used for the prevention and/or treatment of neurodege-
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nerative disorders in mammals, preferably in humans. Furthermore, therapeutic techniques for the treatment of disorders which are associated with the expression of the nucleotide sequence of the nucleic acid according to the present invention may be designed using the above-mentioned agents which are capable
5 of regulating the expression of the nucleotide sequence of the above-defined nucleic acid, e.g. antisense nucleic acids, ribozymes and/or agents for influencing promoter activity. The neurodegenerative disorders are preferably acute and/or chronic neurological and psychological disorders, and may be caused by stroke, parkinson's disease, Alzheimer's disease or other dementias, infections
10 of the CNS and psychiatric disorders associated with disturbances in CNS transmitter systems such as depression and schizophrenia.

In a further preferred embodiment, the pharmaceutical composition according to the present invention further comprises, in addition to the nucleic acid or the
15 vector or the protein or the antibody or the antagonist or the agonist as defined above, one or more other agents having neurotrophic activity. Preferred agents are, e.g., cytokines or functionally active derivatives or parts thereof. Preferred cytokines used in the pharmaceutical composition according to the present invention may be selected from the group consisting of GDF such as GDF-5,
20 GDF-6, GDF-7, GDF-8 and GDF-9, GDNF, TGF such as TGF- α or TGF- β , e.g. TGF- β 1, TGF- β 2 or TGF- β 3, activin A, BMP such as BMP-2, BMP-4, BMP-6, or BMP-7, BMP-11, BMP-12, BDNF, NGF, neurotrophines such as NT-3 or NT-4, EGF, CNTF and FGF such as FGF-2. The term "GDNF" includes GDNF, neurturin and persephin.

25 A further subject of the present invention relates to a diagnostic kit comprising the nucleic acid, the vector, the protein and/or the antibody as defined above, for the detection of neurodegenerative disorders and/or infections of the CNS such as meningitis, e.g. a bacterial meningitis, in mammals, preferably humans.
30 Examples of other neurodegenerative disorders are as defined above.

The figures show:

Fig. 1 Localization of GDF-15 in the CNS. (A) Photographic image of an *in situ* hybridization of an adult rat choroid plexus performed with rat specific GDF-15 antisense-RNA probes. (B) Photographic image of an immunoblot analysis of human cerebrospinal fluid (CSF) under reducing conditions with purified GDF-15 antiserum. (C) RT-PCR of different PO rat brain regions (pons, medulla oblongata, cortex, hippocampus, striatum), dorsal root ganglia (DRG), cultured primary astrocytes (astr.), oligodendroglial cell line OLI-neu (OLI), and cultured oligodendroglial progenitors (O-2A). (D) Immunoblot analysis under native conditions of the corresponding brain areas and cells of (c) with purified GDF-15 antiserum.

Fig. 2 Image of Western blot analysis of GDF-15 in human CSF under reducing conditions. Molecular weight marker (St.). CSF sample of a patient with bacterial meningitis (lane 1). CSF sample of a control patient (lane 2).

Fig. 3 Graphic representation of experiments showing the survival effect of GDF-15 in mesencephalic neuron cultures. Numbers of surviving tyrosine hydroxylase (TH)-immunoreactive neurons of mesencephalic cultures (E15/DIV7) treated with medium only (control), purified lysate from uninfected Sf9 cells-(baculo control), GDF-15 (0.01 to 1 ng/ml) purified from infected Sf9 cell lysate, and GDNF (10 ng/ml). Data are given as mean \pm SEM ($n=3$), P-values derived from Student's t-test are *** $P<0.001$, ** $P<0.01$ for increased survival as compared with control cultures.

Fig. 4 Protective effect of GDF-15 in Fe^{2+} (100 μM) treated cultures. (A) Graphic representation of numbers of surviving TH-immunoreactive neurons of mesencephalic cultures (E15/DIV7) treated with or without Fe^{2+} in medium only (control), in presence of NT-4 (10

ng/ml), and in presence of GDE-15 (10 ng/ml). (B) Graphic representation of the percentage of surviving TH-immunoreactive neurons of mesencephalic cultures (E15/DIV7) treated with Fe^{2+} in medium only (control), in presence of NT-4 (10 ng/ml), and in presence of GDF-15 (10 ng/ml). Values of cultures without addition of iron are set to 100%. Data are given as mean \pm SEM ($n=3$), P-values derived from Student's t-test are $*P<0.05$ for increased survival as compared with control cultures.

10 Fig. 5 (A) cDNA and (B) corresponding amino acid sequence of human pre-pro-mature GDF-15. Nucleotides and amino acids are abbreviated according to the international one letter codes.

15 Fig. 6 (A) cDNA and (B) corresponding amino acid sequence of human mature GDF-15.

The following non-limiting example illustrates the invention:

EXAMPLE

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Identification of GDF-15

25 Using the conserved cystein knot motif of TGF- β -like proteins, a combined approach employing RT-PCR and library screening revealed the full-length cDNA sequence of a novel member of the TGF- β superfamily derived from neurons. The cDNA has the sequence shown in Fig. 5A corresponding to the amino acid sequence shown in Fig. 5B.

30 According to a possible alternative translation start codon which is located 39 nucleotides upstream from the first nucleotide of the sequence shown in Fig. 5A, the corresponding protein may also comprise 13 additional amino acids (MPGQELRTLNGSQ) N-terminal to the sequence shown in Fig. 5B.

The protein, which is named GDF-15, was recombinantly expressed using the baculovirus system. Furthermore, an antibody against a specific peptide derived from the murine and rat C-terminal sequence (HRTDSGVSLQTYDDL) has been developed. Due to the high homology of the corresponding region of the human sequence (QKTDITGVSLQTYDDL), this antibody recognizes also human GDF-15.

Localization of GDF-15 in the CNS

In situ hybridization with GDF-15 antisense RNA probes, RT-PCR as well as Western blot analyses were performed to study the distribution of GDF-15 in the CNS (Fig. 1). *In situ* hybridization revealed signals in neurons, especially Purkinje cells, in the cerebellum, and strong expression in the choroid plexus (Fig. 1A) of newborn and adult rats. RT-PCR and Western blotting of samples taken from different regions of newborn and adult rat brains and peripheral nervous system extended these results by detecting mRNA and protein in pons, medulla oblongata, midbrain, striatum, hippocampus, cortex, and dorsal root ganglia (Fig. 1C, D). Highest levels of mRNA expression were found in the choroid plexus (Fig. 1A). Antibodies raised against the above C-terminal peptide were used for Western blots. Analysis of samples of different brain areas of newborn rats revealed one distinct band at 31 kDa (Fig. 1D). The relative mass of cellular GDF-15 is in good agreement with the theoretical molecular weight of 31 kDa of the pro-protein.

As GDF-15 is abundant in the choroid plexus, the presence of the protein in CSF of healthy human subjects as well as patients with different neurological disorders was also tested. In contrast to the intracellular protein detected in brain samples, CSF samples revealed a single band at about 12 kDa under reducing conditions representing the secreted mature portion of GDF-15. Highest amounts of protein in CSF were seen in patients with bacterial meningitis (Fig. 2). Taken together these data provide evidence that GDF-15, a novel member of the TGF- β superfamily, is widely expressed in various regions of the CNS including CSF and peripheral nervous system. Furthermore, GDF-15 is significantly increased in the CSF of patients with inflammatory neurological disease providing the

opportunity to employ antibodies to GDF-15 as diagnostic tools in neurological disease.

In vitro studies using recombinant human GDF-15

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Using the baculovirus system, the mature part of the human recombinant GDF-15 protein was expressed in Sf9 cells. Western blot showed the monomeric or dimeric form of the recombinant protein under reducing and non-reducing conditions, respectively. Following purification, the protein was tested for its survival effects on rat embryonic midbrain DAergic neurons. Addition of recombinant GDF-15 to cultures of E14 midbrain cells augmented numbers of surviving tyrosine hydroxylase (TH)-positive neurons after 7 days *in vitro* compared to control cultures (Fig. 3). The dopaminotrophic effect of GDF-15 is comparable to the documented survival promoting activity of other members of the TGF- β superfamily and the neurotrophin family (e.g. TGF- β , GDNF-subfamily members, or BDNF). Analysis of midbrain cultures using immunocytochemistry and antibodies to the astrocyte-specific intermediate filament protein GFAP and assays for cell proliferation provided evidence that GDF-15 application did not exert its survival promoting effect through numerically increasing cells and promoting maturation of astrocytes, a well-established source of neurotrophic factors. This provides evidence that GDF-15 affects dopaminergic neurons directly rather than indirectly, as shown for FGF-2 or BMPs.

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In order to investigate whether GDF-15 is also able to protect DAergic neurons against a likely cause of PD, i.e. iron intoxication, its effects on iron-intoxicated mesencephalic neurons was examined (Fig. 4A, B). Exposure of cultures to iron (Fe^{2+}) caused a 80% reduction in neuronal survival compared to untreated control cultures. Cell losses were reduced to 50% when cultures were co-treated with Fe^{2+} and GDF-15. These data strongly suggest that GDF-15 protects DAergic neurons against iron-mediated (oxidative) damage. The data also support the use of GDF-15 as an agent to prevent or slow down neurodegenerative events mediated by free radicals, oxidative stress, mediators and executors of neuronal death programs.

In vivo studies using recombinant human GDF-15

Human recombinant GDF-15 protein was used to study its influence on dopamine depletion induced by 6-hydroxydopamine (6-OHDA) hydrobromide *in vivo*.

5 Adult female Wistar rats (weight range 180 to 200 g) were anaesthetised using ketamine (75mg/kg *i.p.*) and xylazine (15 mg/kg *i.p.*) and placed in a Kopf stereotaxic frame. GDF-15 was used at a final concentration of 2 $\mu\text{g}/\mu\text{l}$ in 10 mM phosphate-buffered saline (PBS) pH 7.4 buffer. Four rats received injections of 20 μg GDF-15 just above the left substantia nigra (SN) and 20 μg GDF-15 into
10 the left lateral ventricle (LV). This was followed immediately by an injection of 6-OHDA hydrobromide (8 μg as the free base in 4 μl 0.9% saline with 0.1% ascorbic acid) into the left medial forebrain bundle (MFB). Four additional rats received 6-OHDA only. Stereotaxic co-ordinates (from bregma and dura; incisor bar at 5.00 mm above the interaural line (Pellegrino et al., A stereotaxic atlas of
15 the rat brain. Plenum Press, New York, 1979) were as follows: AP -3.0, LV +2.5, DV -8.5 for the SN; AP +1.0, LV +1.2, DV -3.5 for the LV; AP -2.2, LV +1.5, DV -7.9 for the MFB.

20 All rats were tested behaviourally at seven days after surgery. Ipsilateral rotations were counted over a 60 min period beginning five min after (+)-amphetamine sulphate administration (5 mg/kg, *i.p.*). The results of these experiments are shown in Tables 1A and B, respectively.

Table 1: Amphetamine rotation data

A: Rotations per minute for 60 min beginning 5 min after amphetamine administration (5 mg/kg *i.p.*)

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Rat no.	Treatment	Rotations per min
1	6-OHDA	13
2	6-OHDA	11
3	6-OHDA	9
4	6-OHDA	11
5	6-OHDA + GDF-15	0
6	6-OHDA + GDF-15	1
7	6-OHDA + GDF-15	2
8	6-OHDA + GDF-15	0

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B: Mean values

Treatment	Rotations per min (mean \pm SD)
6-OHDA	11.0 \pm 1.4
6-OHDA + GDF-15	0.8 \pm 0.8

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All rats displayed the typical features of amphetamine challenge, such as stereotypy and piloerection. Rats which had been treated with 6-OHDA only showed rotation rates of 11.0 ± 1.41 (mean \pm SD), indicating at least 95% depletion of the nigrostriatal pathway (Ungerstedt et al. (1970), Brain. Res., 24, 485-493). In contrast, rats which were also treated with GDF-15 rotated at very low rates (0.75 ± 0.83), showing that this protein effectively prevented 6-OHDA-induced depletion of dopamine in the left striatum.

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Furthermore, in order to confirm that the above prevention of 6-OHDA-induced dopamine depletion in the left striatum is due to a neuroprotective effect of GDF-15 on neurons in the SN, the SN pars compacta (SNpc) was analysed immuno-

cytochemically. At ten days after surgery, all rats were terminally anaesthetised with chloroform/ether and perfused intracardially with 200 ml of cold 0.1 M phosphate-buffered saline, pH 7.4 (PBS) containing 500 Units heparin, followed by 300 ml freshly-prepared 4% paraformaldehyde in PBS. Brains were removed and placed in 4% paraformaldehyde in PBS overnight, cryoprotected in 30% sucrose in PBS and then frozen. Serial 30 μ m coronal cryosections through the SNpc were cut and stained immunocytochemically for tyrosine hydroxylase (TH). Sections were incubated in blocking solution (3% normal goat serum, 0.2% Triton X-100 in PBS) overnight at 4°C, then in a 1:2000 solution of rabbit antiserum directed against TH (Affiniti Labs, U.K.) in blocking solution overnight at 4°C. Sections were washed five times in PBS containing 0.02% Triton X-100, then incubated in a solution of 1:1000 horse radish peroxidase-linked anti-rabbit IgG (Vector Labs) overnight at 4°C. After washing as before, TH immunostaining was visualised using 3,3'-diaminobenzidine as the chromogen. Sections were mounted onto gelatinised slides, dehydrated in alcohol, cleared in xylene and mounted in DePeX® (Bioproducts, Heidelberg, Germany). TH-immunoreactive neurons were counted in the SNpc on both sides of the brain at each of three levels; -2.8, -3.0, -3.2, with respect to bregma (Pellegrino et al., A stereotaxic atlas of the rat brain. Plenum Press, New York, 1979).

Counts of TH-positive neurones in the SNpc measured at three individual levels are shown in Table 2A and the mean values for each rat are given in Table 2B, respectively. The overall mean values for the 6-OHDA-treated ($n = 4$) and for the rats which were co-treated with 6-OHDA and GDF-15 ($n = 4$) are shown in Table 2C. The results show that the co-treatment of the rats with 6-OHDA and GDF-15 led to a 10-fold increase in the count of TH-positive neurons in the left striatum compared to the treatment with 6-OHDA alone. Therefore, GDF-15 prevents 6-OHDA-induced depletion of dopamine in the left striatum due to its strong neuroprotective effect on TH-immunoreactive neurons.

Table 2: TH-immunocytochemistry data

A: Counts of TH-positive neurones in substantia nigra pars compacta at three levels; -2.8, -3.0 and -3.2, relative to bregma (according to Pellegrino et al., A stereotaxic atlas of the rat brain. Plenum Press, New York, 1979)

Rat no.	Treatment	TH counts (-2.8)			TH counts (-3.0)			TH counts (-3.2)		
		Right	Left	L/R (%)	Right	Left	L/R (%)	Right	Left	L/R (%)
1	6-OHDA	102	6	5.9	121	8	6.6	125	11	8.8
2	6-OHDA	114	11	9.6	117	10	8.5	114	12	10.5
3	6-OHDA	98	3	3.1	104	4	3.8	106	9	8.5
4	6-OHDA	99	3	3.0	112	7	6.3	97	6	6.2
5	6-OHDA + GDF-15	107	69	64.5	111	73	65.8	114	81	71.1
6	6-OHDA + GDF-15	110	71	64.5	109	65	59.6	120	84	70.0
7	6-OHDA + GDF-15	114	78	68.4	123	101	82.1	126	97	77.0
8	6-OHDA + GDF-15	115	80	69.6	118	95	80.5	118	89	75.4

B: Mean values of individual rats

Rat no.	Treatment	TH counts (mean \pm SD)		
		Right	Left	L/R (%)
1	6-OHDA	116.0 \pm 10.0	8.3 \pm 2.1	7.1 \pm 1.2
2	6-OHDA	115.0 \pm 1.4	11.0 \pm 0.8	9.5 \pm 0.8
3	6-OHDA	102.7 \pm 3.4	5.3 \pm 2.6	5.1 \pm 2.4
4	6-OHDA	102.7 \pm 6.6	5.3 \pm 1.7	5.2 \pm 1.5
5	6-OHDA + GDF-15	110.7 \pm 2.9	74.3 \pm 5.0	67.1 \pm 2.9
6	6-OHDA + GDF-15	113.0 \pm 5.0	73.3 \pm 7.9	64.7 \pm 4.2
7	6-OHDA + GDF-15	121.0 \pm 5.1	92.0 \pm 10.0	75.8 \pm 5.7
8	6-OHDA + GDF-15	117.0 \pm 1.4	88.0 \pm 6.2	75.2 \pm 4.5

C: Mean values of 6-OHDA-treated rats and mean values after co-treatment with 6-OHDA plus GDF-15

Treatment	TH counts (mean \pm SD)		
	Right	Left	Left/Right (%)
6-OHDA	109.1 \pm 6.4	7.5 \pm 2.4	6.7 \pm 1.8
6-OHDA + GDF-15	115.4 \pm 3.9	81.9 \pm 8.2	70.7 \pm 4.9

In conclusion, a novel neurotrophic molecule derived from neuron cells belonging to the TGF- β superfamily, GDF-15, is provided and functionally characterized.

In the nervous system, GDF-15 mRNA and protein can be detected, e.g. in midbrain, striatum and in cortex, but highest levels of the mRNA and the protein are found in the choroid plexus and spinal fluid (CSF), respectively. Interestingly, levels of protein in CSF are increased in certain neurological disorders, e.g. in patients with bacterial meningitis. In order to elucidate its functions, the mature form of human GDF-15 was recombinantly expressed using a baculovirus expression system. Expression resulted in the synthesis of the biologically active dimeric form of the protein. *In vitro* experiments using dissociated cell cultures of embryonic rat midbrain neurons revealed that GDF-15 can act as a neurotrophic factor for DAergic midbrain neurons which degenerate in Parkinson's disease (PD). Furthermore, GDF-15 is able to protect these neurons against intoxication by iron, which may be causal to PD.

Furthermore, experiments using adult rats as a model system showed that GDF-15 effectively prevents 6-hydroxydopamine-induced neuron death in the substantia nigra *in vivo*.

Therefore, it can be concluded that GDF-15 has important functions in the developing, mature, and lesioned brain involving options to use GDF-15 for the treatment and diagnosis of acute and chronic neurological and psychological disorders, such as stroke, Alzheimer's disease and other demetias, and psychiatric disorders associated with disturbances in CNS transmitter systems.



July 28, 1999

Müller-Boré & Partner

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Applicant: Biopharm Gesellschaft zur biotechnologischen Entwicklung von
Pharmaka mbH

"Neuroprotective properties of GDF-15 in vitro and in vivo"

Our Ref: B 1951EU - py / js

EPO-Munich
52

29. Juli 1999

Claims

1. A nucleic acid containing a nucleotide sequence encoding the primary amino acid sequence of a TGF- β -like protein or a functionally active derivative or part thereof which is derived from neurons and glial cells and which has a neurotrophic effect on DAergic neurons.
2. The nucleic acid of claim 1, consisting substantially of deoxyribonucleotides and/or ribonucleotides and/or modified nucleotides.
3. The nucleic acid of claim 1 or 2, wherein the neuron and glial cells are of mammalian origin.
4. The nucleic acid according to anyone of claims 1 to 3, wherein the protein protects against neurodegenerative events.
5. The nucleic acid of claim 4, wherein the neurodegenerative event is mediated by oxidative damage and/or free radical damage and/or mediators and/or executors of neuronal death programs.
6. The nucleic acid according to claim 5, wherein the mediators of the free radical damage are selected from the group consisting of iron, NO donors, and other free radical donors, and the mediators and executors of neuronal death programs are selected from the group consisting of caspases and pro- and anti-apoptotic members of the bcl-2 family.

- 5
7. The nucleic acid according to anyone of claims 1 to 6, comprising at least the nucleotide sequence shown in Fig. 5A or the nucleotide sequence shown in Fig. 6A or nucleotides 40 to 333 of the nucleotide sequence shown in Fig. 6A or mutants thereof leading to the expression of functionally active polypeptides.
- 10
8. A vector containing at least the nucleic acid according to anyone of claims 1 to 7.
9. A host organism containing at least the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8.
- 15
10. A protein encoded by the nucleic acid according to anyone of claims 1 to 7.
11. An antibody or a functional fragment thereof directed against the protein of claim 10.
12. An antagonist directed to the protein of claim 10.
- 20
13. An agonist as a substitute for the protein of claim 10.
14. A method for the production of the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8 or the protein of claim 10, comprising the steps of:
- 25
- (a) cultivating the host organism of claim 9 in a suitable medium under suitable conditions; and
- (b) isolating the desired product from the medium and/or the host organisms.
- 30
15. A pharmaceutical composition comprising the nucleic acid according to anyone of claims 1 to 7 or the vector of claim 8 or the protein of claim 10 or the antibody of claim 11 or the antagonist of claim 12 or the agonist of

claim 13, optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

5 16. The pharmaceutical composition of claim 15 for the prevention and/or treatment of neurodegenerative disorders in mammals.

17. The pharmaceutical composition of claim 16, wherein the mammal is a human.

10 18. The pharmaceutical composition of claim 16 or 17, wherein the neurodegenerative disorders are selected from the group of acute and/or chronic neurological and psychological disorders.

15 19. The pharmaceutical composition of claim 18, wherein the neurological and psychological disorders are caused by stroke, parkinson's disease, Alzheimer's disease or other dementias, infections of the CNS and psychiatric disorders associated with disturbances in CNS transmitter systems.

20 20. The pharmaceutical composition of claim 19, wherein the psychiatric disorders are selected from the group consisting of depression and schizophrenia.

25 21. The pharmaceutical composition according to anyone of claims 15 to 20 further comprising one or more agents having neurotrophic activity or functionally active derivatives or parts thereof.

22. The pharmaceutical composition of claim 21, wherein the agent is a cytokine.

30 23. The pharmaceutical composition of claim 22, wherein the cytokine is selected from the group consisting of GDF, GDNF, TGF, activin A, BMP, BDNF, NGF, EGF, CNTF and FGF.

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24. A diagnostic kit comprising the nucleic acid according to anyone of claims 1 to 7 and/or the vector of claim 8 and/or the protein of claim 10 and/or the antibody of claim 11 for the detection of neurodegenerative disorders and/or infections of the CNS in mammals.

5

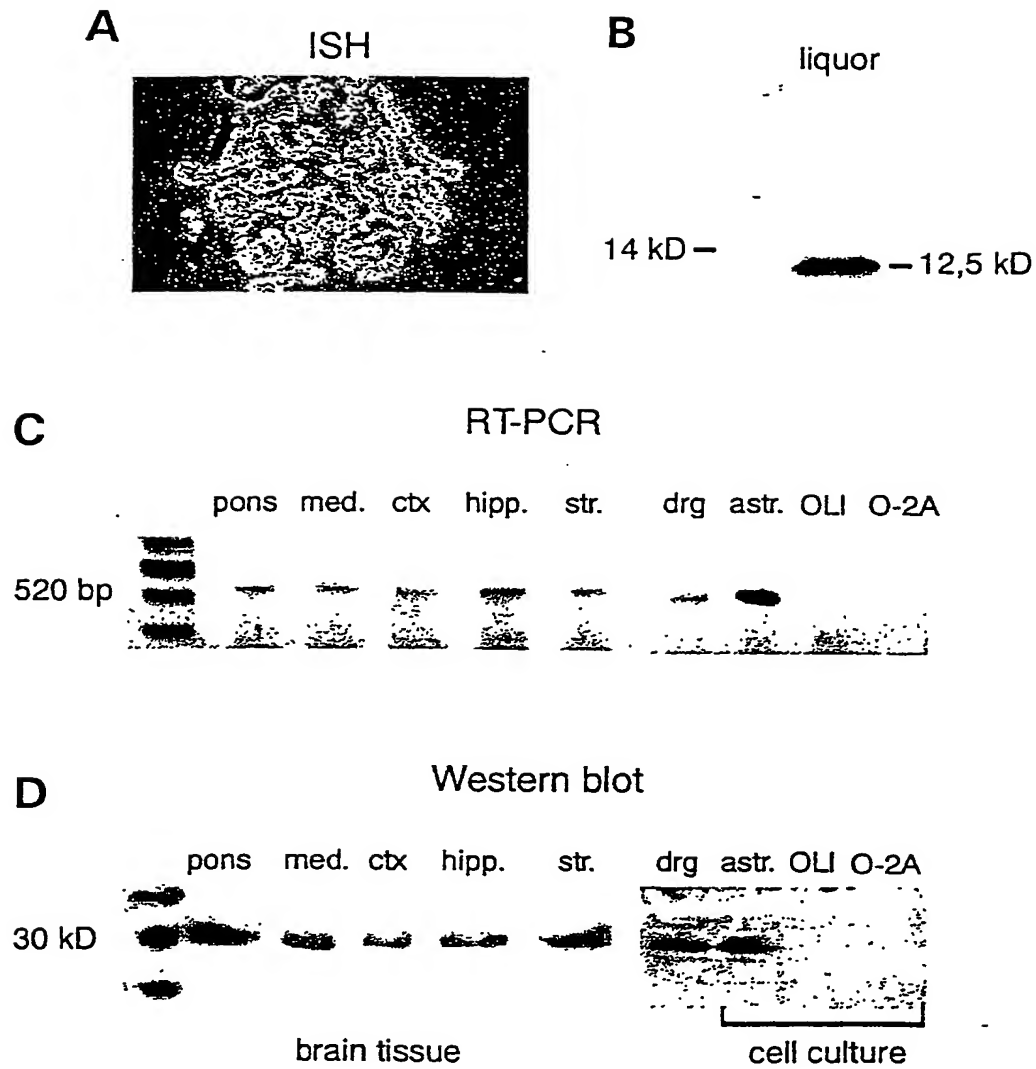
25. The diagnostic kit of claim 24, wherein the infection is a meningitis.
26. The diagnostic kit of claim 24 or 25, wherein the mammal is a human.

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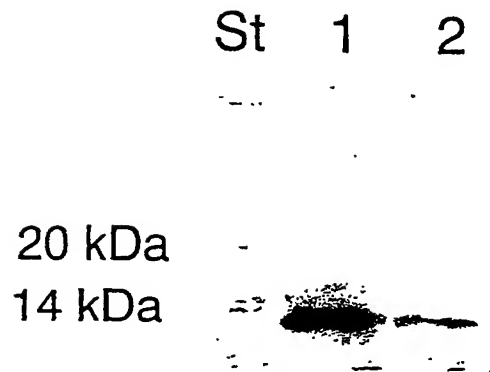
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Fig. 1



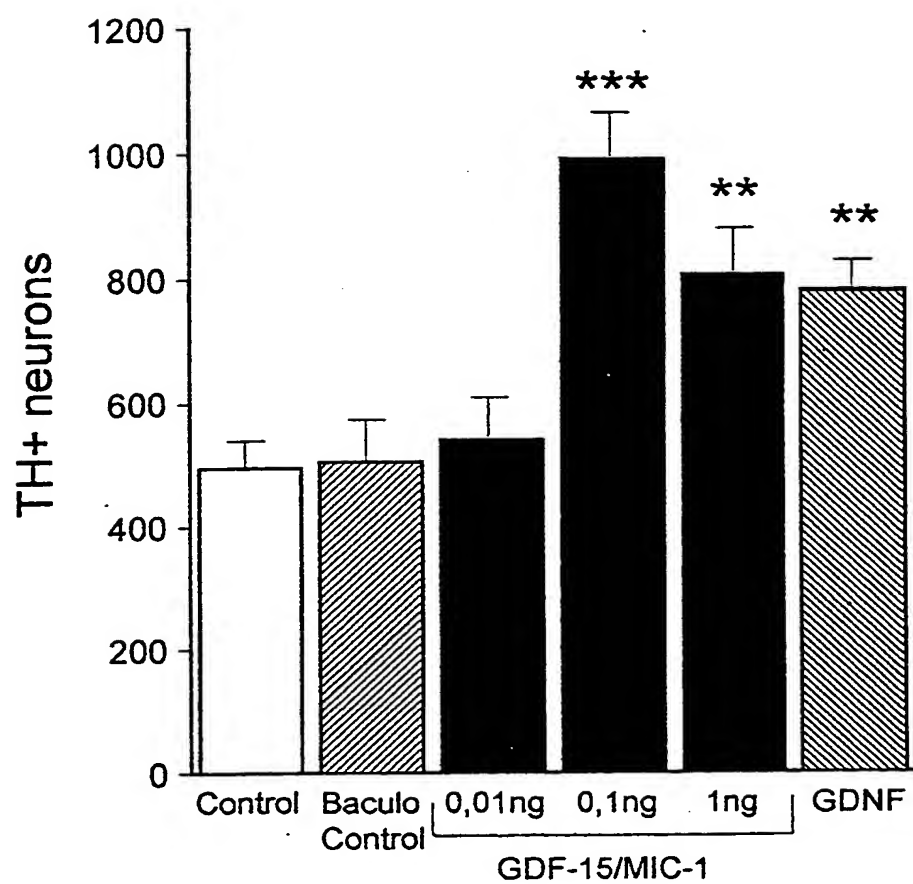
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Fig. 2



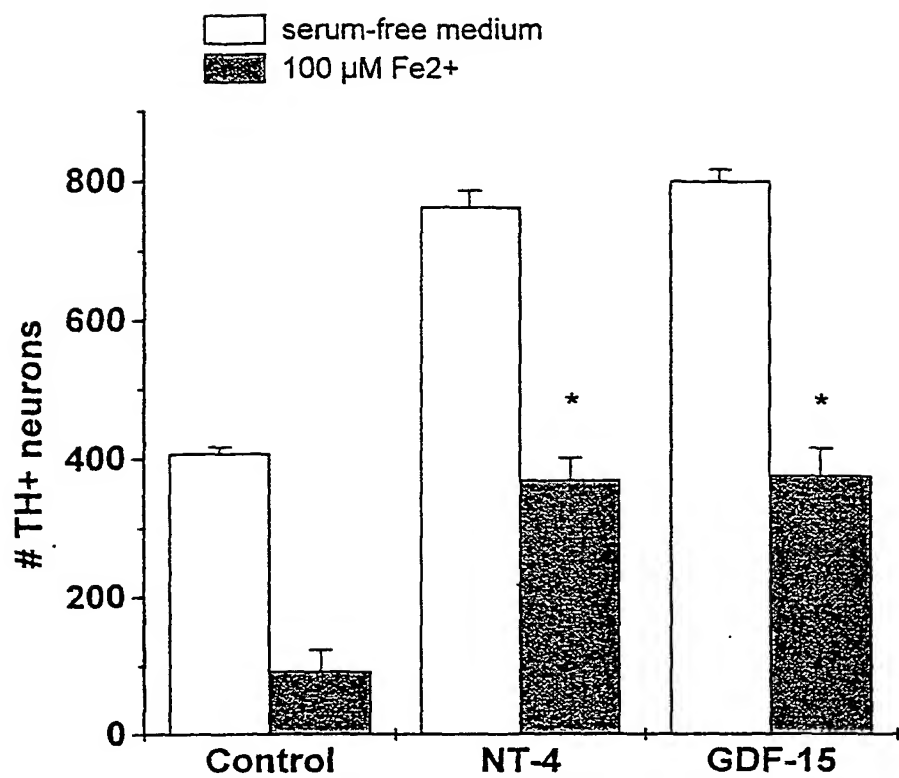
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Fig. 3



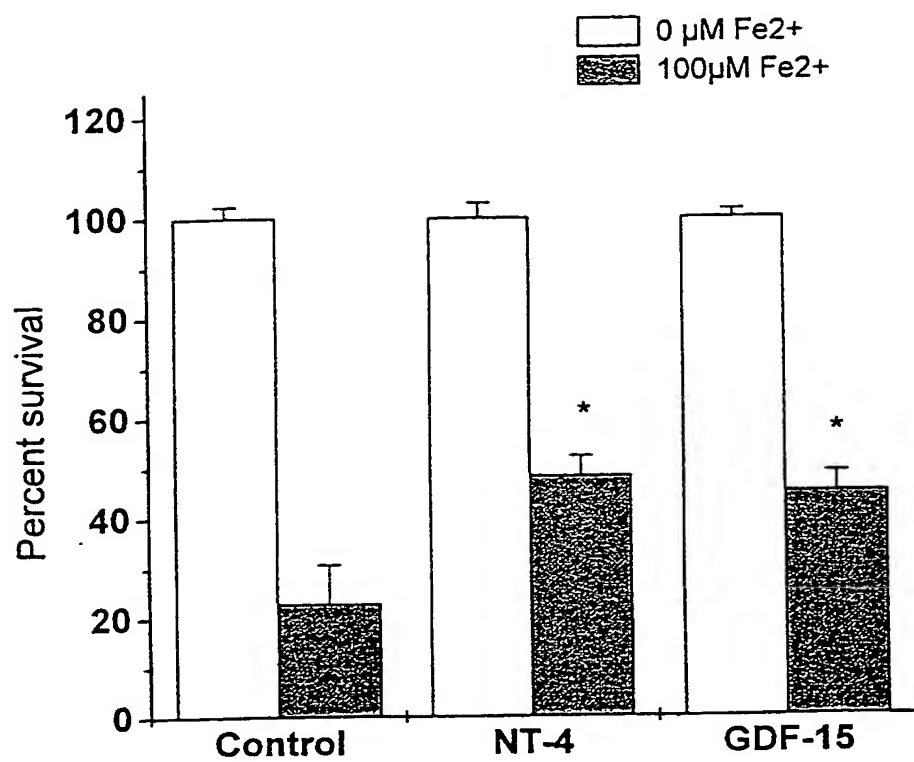
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Fig. 4A



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Fig. 4B



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Fig. 5A

cDNA hGDF-15 (pre-pro-mature)

ATGCTCCTGGTGTGCTGGTGTCTCTCGTGGCTGCCGCATGGGGGCGCCCTGTCTCTGGCCG
AGGCGAGCCGCGCAAGTTTCCCGGGACCCTCAGAGTTGCACTCCGAAGACTCCAGATTCCG
AGAGTTGCGGAAACGCTACGAGGACCTGCTAACCAGGCTGCGGGCCAACCAGAGCTGGGAA
GATTCGAACACCGACCTCGTCCCGGCCCTGTCAGTCCGGATACTCACGCCAGAAGTGCGGC
TGGGATCCGGCGGCCACCTGCACCTGCGTATCTCTCGGGCCGCCCTTCCCGAGGGGGCTCCC
CGAGGCCTCCCGCCTTCACCGGGCTCTGTTCCGGCTGTCCCCGACGGCGTCAAGGTCGTGG
GACGTGACACGACCGCTGCGGCGTCAGCTCAGCCTTGCAAGACCCCGAGCGCCCGCGCTGC
ACCTGCGACTGTGCGCGCCGCCGTCGAGTCGGACCAACTGCTGGCAGAATCTTCGTCCGC
ACGGCCCCAGCTGGAGTTGCACTTGCGGCCGCAAGCCGCCAGGGGGCGCCGAGAGCGCGT
GCGCGCAACGGGGACGACTGTCCGCTCGGGCCCGGGCGTTGCTGCCGTCTGCACACGGTCC
GCGCGTCGCTGGAAGACCTGGGCTGGGCGGATTGGGTGCTGTGCCACGGGAGGTGCAAGT
GACCATGTGCATCGGCGCGTGCCCGAGCCAGTTCGGGGCGGCAAACATGCACGCGCAGATC
AAGACGAGCCTGCACCGCCTGAAGCCCGACACGGTGCCAGCGCCCTGCTGCGTGCCCGCCA
GCTACAATCCCATGGTGCTCTTACAAAAGACCGACACCGGGGTGTCGCTCCAGACCTATGA
TGACTTGTTAGCCAAAGACTGCCACTGCATATGA

Fig 5B

protein hGDF-15 (pre-pro-mature)

MLLVLLVLSWLPHGGLSLAEASRASFPGPSELHTEDSRFRELKRYE...
DLLTRLRANQSWEDSNTDLVPAPAVRIITPEVRLGSGGHLHLRISRAALPEGLPEASRLHR
ALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLSPFPSQSDQLLAESSARPQLELH
LRPQAARGRRRARARNGDHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMCIGAC
PSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYNPMVLIQKTDGTGVSLQTYDDLAKDC
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Fig. 6A

cDNA hGDF-15 (mature)

GCGCGCAACGGGGACGACTGTCCGCTCGGGCCCGGGCGTTGCTGCCGTCTGCACACGGTCC
GCGCGTCGCTGGAAGACCTGGGCTGGGCCGATTGGGTGCTGTCGCCACGGGAGGTGCAAGT
GACCATGTGCATCGGCGCGTGCCCCGAGCCAGTTCCGGGCGGCAAACATGCACGCGCAGATC
AAGACGAGCCTGCACCGCCTGAAGCCCGACACGGTGCCAGCGCCCTGCTGCGTGCCCGCCA
GCTACAATCCCATGGTGCTCTTACAAAAGACCGACACCGGGGTGTCGCTCCAGACCTATGA
TGACTTGTTAGCCAAAGACTGCCACTGCATATGA

Fi.g 6B

protein hGDF-15 (mature)

ARNGDHCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMCIGACPSQFRAANMHAQI
KTSLHRLKPDTVPAPCCVPASYNPMVLIQKTDGTGVSLQTYDDLLAKDCHCI



Applicant: Biopharm Gesellschaft zur biotechnologischen Entwicklung von
Pharmaka mbH
"Neuroprotective properties of GDF-15 in vitro and in vivo"
Our Ref: B 1951EU - py / js

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Abstract

The present invention relates to a transforming growth factor-beta (TGF- β)-like protein which is derived from neurons and glial cells, and which has a neurotrophic effect on dopaminergic (DAergic) neurons, to nucleic acids coding for the protein, to a vector containing the nucleic acids, to host organisms containing the nucleic acids or the vector, to antibodies directed against the protein, to methods for the production of the nucleic acids, the vector or the protein, to a pharmaceutical composition for the treatment of neurodegenerative disorders in mammals and to a diagnostic kit for the detection of said disorders.

